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Dye Ligands

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Introduction

Dyes employed in protein and enzyme purification are synthetic hydrophilic molecules bearing a reactive, usually a chlorotriazine, moiety by which they can easily be attached to various polymeric supports. Among the various known reactive dye ligands, Cibacron Blue 3GA or F3GA (CB3GA, **Figure 1**A), an *ortho*-isomer of CI Reactive Blue 2, has attracted most attention from biotechnologists in protein purification. The foundations of the important role of CB3GA may well be attributed to pure historical accident; an anomalous gel permeation chromatography run of pyruvate kinase when using blue dextran as a void volume marker. It was later found that the blue chromophore, CB3GA, was responsible for binding the enzyme thus leading to co-elution of enzyme and blue dextran. As with many critical but unexpected discoveries, the importance and breadth of applications were hardly appreciated in those early days. Since then, CB3GA and other triazinyl dyes have been immobilized on to various supports and used in the affinity purification of many proteins and enzymes.

Development of Dye Ligands and Dye Affinity Adsorbents

The originally exploited dyes were commercial textile chlorotriazine aromatic polysulfonated molecules which when attached to appropriate supports, usually bearing hydroxyl groups, yield dye affinity adsorbents. The range of shades of commercial dyes derives primarily from anthraquinone, azo and phthalocyanine chromophores bonded to suitable reactive functions such as triazinyl and other mainly polyhalogenated heterocyclics. Anthraquinone dyes produce blue and the phthalocyanines turquoise shades. Green dyes contain mixed anthraquinone–stilbene, anthraquinone–azo or phthalocyanine–azo structures, whereas most other shades are derived from the azo class.

Unlike most biological affinity adsorbents, the stability of dye affinity adsorbents is usually limited only by the support itself. Dyes offer clear advantages over biological ligands, in terms of economy, ease of immobilization, safety, stability and adsorbent capacity. The main drawback of textile dyes is their moderate selectivity during the protein-binding process. In spite of this, the overall size, shape and distribution of ionic and hydrophobic groups enable dyes to interact with the binding sites(s) of proteins sometimes fairly specifically, as for example, with the nucleotide-binding site of several dehydrogenases, kinases, and several nucleotide-recognizing enzymes. The dye–protein interaction should not be compared to a simple ion exchange type since binding is frequently possible at pHs greater than the p*I* of the targeted protein. Furthermore, dissociation of the dye–protein complex is often achieved specifically by competing ligands, suggesting interaction with the protein at discrete sites. The view is supported by chromatographic, kinetic, inactivation, affinity labelling and spectra difference studies.

The last few years have seen a novel approach for tackling the problem of dye selectivity, signalling the

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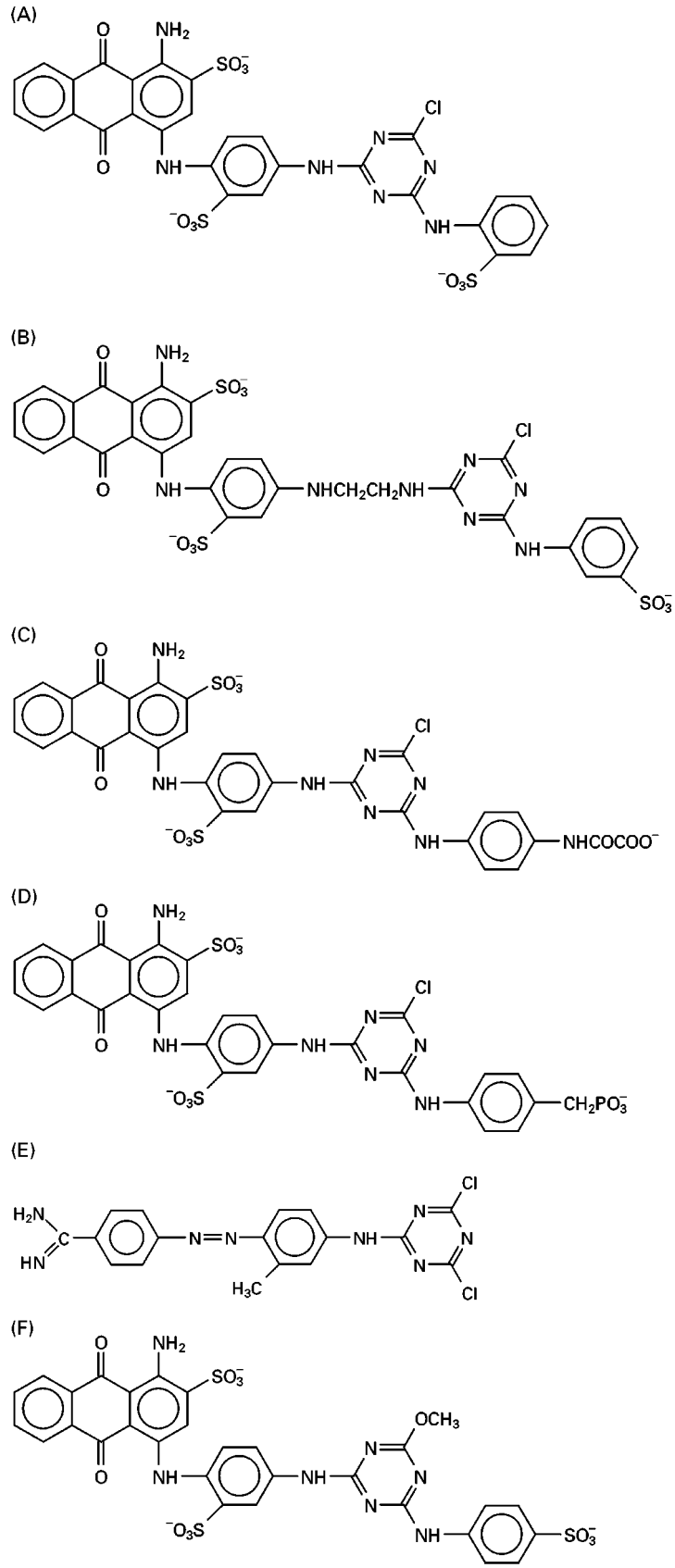

Figure 1 Structures of the parent textile dye Cibacron Blue 3GA (CB3GA), (A) the three blue (B)–(D) and one yellow (E) biomimetic dyes, and the blue dye (F) for selective LDH precipitation.

beginning of a new era in affinity bioseparation. This approach is based on a generation of the so-called biomimetic dyes. By employing molecular modelling techniques, it is possible to redesign the parent dye (e.g. CB3GA) or design *de novo* a new dye in such a way that the resulting biomimetic dye mimics naturally occurring biological ligands, thus displaying increased selectivity for the targeted enzyme. Figure 1 shows the structures of the parent dye CB3GA (A) and four biomimetic dyes (B–E). For the case of the NAD^+-dependent enzyme alcohol dehydrogenase (ADH), having used molecular modelling to compare the conformations that NAD^+ and CB3GA adopt during binding to the enzyme, it was possible to propose the biomimetic structure of **Figure 1**(**B**) that exhibits increased affinity for ADH as compared to CB3GA. Recently a family of anthraquinone biomimetic dyes has been designed specifically for (keto)carboxyl-group-recognizing enzymes. Each member of this dye family is composed of two enzyme-recognition moieties (e.g. **Figure 1C**). The terminal biomimetic moiety bears a (keto)carboxyl structure linked to the triazine ring, thus mimicking substrates of the targeted enzyme, e.g. L-malate dehydrogenase (MDH). The chromophore anthraquinone moiety remains unchanged and the same as that of the parent dye (Figure 1A), recognizing the nucleotide-binding site of MDH. Members of this dye family show increased affinity for the targeted enzymes and have been designed for other enzymes as well, e.g. L-lactate dehydrogenase (LDH). In the case of the orthophosphate ester hydrolase alkaline phosphatase, a biomimetic dye was designed by substituting the terminal 2-aminobenzene sulfonate of CB3GA for a 4-aminobenzene phosphonate ring (**Figure 1D**). The corresponding dye adsorbent offers an impressive purification of calf intestinal alkaline phosphatase of over 300-fold in a single chromatography step. The biomimetic structure of **Figure 1**(**E**) is a benzamidino-cationic yellow dye designed for a different application. The cationic dye bears a guanidino group, the same as the potent trypsin inhibitor benzamidine. When the dye is immobilized, it is able to separate the two main proteolytic constituents of crude pancreatic extract, trypsin and chymotrypsin, since only the former enzyme is adsorbed. The use of biomimetic dyes is expected to increase and help towards simplifying enzyme purification problems.

Development of the Techniques

Dyes, whether conventional or biomimetic, have been exploited by different affinity bioseparation techniques for protein and enzyme purification, as described below.

Affinity Chromatography

Reactive chlorotriazinyl dyes are easily and safely immobilized on agarose and other polyhydroxylic supports (e.g. cellulose, polyacrylates) or, less often, on amino-group-bearing supports, after nucleophilic substitution in alkaline environment. Alternatively, a diaminoalkane (e.g. 1,6-diaminohexane) spacer molecule can be chemically attached to the chlorotriazine ring of the dye, before the resulting conjugate is immobilized on various pre-activated polyhydroxylic supports. Although chemical immobilization of reactive dyes on polyhydroxylic supports is the most straightforward and widely used technique, perhaps the most unusual one is immobilization via adsorption. For example, it is possible, first, to chemically attach to the reactive dye a hydrophobic 1H,1H-pentadecafluorooctylamine tail by nucleophilic substitution. Then, the dye–tail conjugate can be physically adsorbed on to hydrophobic fluorocarbon particles. Hydrophobic fluorocarbon supports can be transformed into surface-hydrophilic materials. In this case, hydrophobic perfluorocarbon tails are chemically attached to hydrophilic polyvinylalcohol (PVA). Afterwards, the PVA–tail conjugate is physically adsorbed on to the hydrophobic surface of Teflon® particles. Therefore, the transformed Teflon® surface is hydrophilic and rich in hydroxyl groups through which reactive dyes can be immobilized.

At the end of the immobilization reaction, the coloured adsorbent is washed to remove free dye, packed in a chromatography column, and equilibrated with the appropriate buffer for protein purification. The sample is applied and nonadsorbed proteins are washed off, before the desired protein is desorbed (eluted) by changing the composition of the buffer eluent. This is realized either by nonspecific desorption techniques (i.e. change of the ionic strength or pH) or by specific desorption techniques, for example, introducing competing substances (i.e. substrates, inhibitors, metal chelators) in order to selectively perturb the enzyme–dye complex on the column.

In the development of a protein purification protocol, some form of adsorbent screening exercise should be performed as the first step. The adsorbent exhibiting the highest purification factor and recovery for the targeted protein on the one hand, and on the other hand the adsorbent showing no adsorption of the protein of interest but good total protein adsorption, are promising candidates for employment in

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positive and negative affinity chromatography, respectively.

Although beaded porous polyhydroxylic supports have been used for decades in biochromatography, rapid mass transfer has only been achieved recently thanks to two novel chromatography supports. The 'flow-through particles', which are exploited in perfusion chromatography, have pores wide enough (0.6–0.8 μm) to allow convective flow through them. Smaller diffusive pores along the throughpore channels of these particles provide a high adsorption area with diffusion path lengths less than 1 μm. These particles reduce process time by improving mass transfer. An alternative for the same purpose is offered by hyperdiffusion chromatography; this uses rigid particles with their entire pore volume filled by a homogeneous flexible soft hydrogel where the affinity ligands are immobilized. The proteins can diffuse freely through the hydrogel network and interact with the ligand.

High Performance Affinity Chromatography

When the support is made of noncompressible, spherical particles of small diameter (e.g. 5–20 μm) and narrow size distribution (e.g. 0.2–2.0 μm) the technique is termed high performance affinity chromatography (HPAC). Silica-based supports have been used with dyes for HPAC, although silica has serious drawbacks: poor chemical stability in an alkaline environment; the need for derivatization with organofunctional silanes prior to dye immobilization; and relatively small pore size. In spite of its problems, silica is still used in HPAC mainly on an analytical scale. Synthetic high performance polyhydroxylic particles probably offer a better alternative to silica for HPAC. The chemistry of support activation and dye immobilization is generally the same as that for softer materials, as described earlier.

A different concept for column packings was introduced by the following two materials: deformed nonporous high performance agarose; and nonporous fibres. The former support consists of 12–15 μm diameter nonporous beads which, when compressed, are claimed to result in superior resolution at high flow rates. The latter support consists of nonporous fibreform quartz material of mean diameter 0.5 μm. This material has first to be silylated prior to proceeding with ligand immobilization. The above materials are potentially suitable for use with dye ligands.

Centrifugal Affinity Chromatography

Centrifugal affinity chromatography combines the high flow rate, created by centrifugation force, with the specificity of affinity chromatography. It is an alternative to closed column chromatography (i.e. columns with adapters at their ends minimizing dead volumes) which usually but not always requires elaborate instrumentation. This technique has demonstrated its usefulness in the removal of albumin from human serum using immobilized CB3GA, and in the rapid screening of a large number of immobilized dyes for binding goat IgG.

Heterobifunctional Ligand Affinity Chromatography

The principle of heterobifunctional affinity ligand chromatography requires that a soluble heterobifunctional affinity ligand possessing two different affinity moieties is available. This alone may be a drawback for the technique, when considering factors such as labour and cost. One moiety is destined to interact in solution with the targeted protein for purification, while the second moiety should be able to recognize and bind to a suitable affinity adsorbent. A model system based on this principle has been developed for purifying lactate dehydrogenase (LDH). In this case, the soluble heterobifunctional ligand consisted of the dye CB3GA chemically linked to soya bean trypsin inhibitor, whereas immobilized trypsin is used in an affinity column for binding the soluble complex trypsin inhibitor · CB3GA-LDH.

Continuous Affinity Recycle Extraction

In continuous affinity recycle extraction, instead of packing affinity adsorbent particles in a fixed bed, adsorbent/liquid contact is performed in well-mixed tanks. The adsorbent is continuously recirculated between two tanks. Each tank consists of two concentric cylindrical vessels. The wall of the interior vessel is made of a supported filter. The filter porosity is such that it does not allow passage of the adsorbent but only of the liquid stream and its soluble contents, so they can flow through freely. The affinity adsorbent is placed in the space between the concentric cylinders and is continuously recirculated between the two tanks. In one tank there is continuous adsorption of protein to the affinity adsorbent, whereas unbound materials pass through the filter to waste or recycle. In the second tank there is continuous desorption of product from the adsorbent, through the filter, to the collection apparatus. This technique can be applied to dye affinity adsorbents, particularly on a large scale.

Aqueous Two-phase Affinity Partitioning

Affinity partitioning is an inexpensive alternative to affinity chromatography-based purification

technologies, and it is also suitable for large scale application. When aqueous solutions of two high molecular weight polymers, e.g. dextran and polyethylene glycol (PEG), are mixed and then left to settle, depending on the relative molecular mass of the polymers, their concentration and temperature, two phases may be formed: an upper PEG-rich phase and a bottom dextran-rich phase. Under appropriate conditions, many proteins partition to the bottom dextran-rich phase. However, when some of the PEG of the upper phase is replaced by a suitable dye–PEG conjugate, the targeted protein may bind to the dye–PEG conjugate and selectively partition to the upper PEG-rich phase, thus, leading to some purification. An important advantage of affinity partitioning is that the technique can be exploited at the first stage of a purification process, immediately after cell disintegration, without need of centrifugation for prior debris removal. Dye–PEG conjugates have been employed in two-phase affinity systems for partial purification of numerous enzymes, especially those recognizing nucleotides.

Membrane Affinity Filtration

Chemical attachment of the affinity ligand to a filtration membrane provides the principle of the membrane affinity filtration technique. The role of the filter component can be taken by a hollow-fibre microporous membrane where the ligand is chemically attached. The feed stream moves along the interior of the fibre whose pores are large enough (0.5–1.0 μm) to permit convective flow through its body. While binding of the targeted protein to the immobilized ligand occurs without diffusion limitations, unbound substances pass through the fibre body to its outer surface. The main advantage offered by this technique, which is important to industrial-scale purification, is that there is no need for solids removal from the feed stream after cell disintegration. In fact, liquid–solid separation and affinity purification are combined and performed simultaneously when using this technique. Affinity membranes permit rapid processing of large volumes, sometimes even immediately after cell harvesting or disintegration, thus eliminating concentration and partial purification stages. Membrane systems other than hollow fibres have been developed, for example membrane discs and modules of flat membrane sheets bearing immobilized dyes. Such materials are effective tools in protein downstream processing.

Affinity Cross-flow Ultrafiltration

The availability of an affinity macroligand capable of reversible and selective binding of the targeted protein is essential for applying the technique of affinity cross-flow ultrafiltration. A macroporous membrane is also required which allows passage of the unwanted substances and the targeted protein, but not of the affinity macroligand. When the sample is filtered in the presence of the macroligand, the protein of interest is retained by adsorbing on to the macroligand. During the washing step the targeted protein is desorbed, passes through the membrane and is collected. However, the macroligand will remain retained by the membrane and can therefore be recycled and reconditioned. As with membrane affinity filtration, affinity cross-flow ultrafiltration offers rapid mass transfer, high throughput, and is capable of processing unclarified and viscous materials.

Affinity Precipitation

Affinity precipitation is a bioseparation technique whereby an insoluble network is formed by cross-linking a multifunctional affinity ligand with the protein of interest, thus resulting in selective precipitation of the targeted protein from solution. In principle, such a ligand should be composed of three recognition sites which are able to bind the protein simultaneously. Furthermore, the targeted protein should possess enough ligand-binding sites for the formation of a network. This technique offers certain advantages which are particularly important for large scale purification: no need for solid supports, high ligand utilization, low capital cost, and the possibility of sequential precipitation. True affinity precipitation has been demonstrated for only a few purification cases. An impressive example is the dye structure of **Figure 1**(**F**). This nonreactive molecule selectivity precipitates and purifies lactate dehydrogenase to homogeneity from crude extract of rabbit muscle. Affinity precipitation has been claimed to occur also with bis-Cibacron Blue 3GA but this technique has not found the anticipated attention.

Instrumentation

Of the techniques described above, dye affinity chromatography and high performance dye affinity chromatography are the main ones employing sophisticated instrumentation. Closed columns (i.e. columns with adapters at both ends) packed with affinity adsorbents can be run and studied with the highest possible precision on automated and computerized chromatography instruments. Such instruments are, for example, the FPLC® System of Pharmacia Biotechnology, and the Waters 650E Advanced

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Protein Purification System. Of course, the systems can be used also with nonaffinity chromatography materials. The FPLC® system (pressure limit 4 MPa/580 psi, flow rate range 0.02–8.3 mL min^{-1}) is composed, typically, of a chromatography controller, which offers the facility for connecting to a computer, and controls the rest of the system components: two dual-syringe high precision pumps; a mixing device; an injection motor valve; a sampling loop device to introduce, without dilution, volumes of up to 50 mL; an absorbance detector system with a recorder to monitor column effluents; and a fraction collector with a solenoid valve. Additional motor valves can be used for selecting different liquid phases and columns. The same company also manufactures the BioPilot® system, made especially for exploiting large column beds and processing large sample volumes; it has the similar basic philosophy as the FPLC®. The system offers a flow rate range 0.5–100 mL min^{-1} at pressures up to 2 MPa/290 psi, and sample volumes up to 150 mL introduced via a loop device. A money-saving alternative to FPLC® offered by Pharmacia Biotechnology, is the GradiFrac™ which incorporates, in a one piece instrument, a fraction collector and a gradient maker. It requires additionally a peristaltic pump, an absorbance monitor, an injection valve, a mixing device, a solenoid valve to form the gradient, and a fraction collector with a solenoid valve. The Waters system is also a nonmetallic liquid chromatograph, operating at flow rate ranges of 0.1–45 mL min^{-1} or 0.5–80 mL min^{-1}, depending on the pump heads used, having a pressure limit of 750 psi. It is composed of a chromatography and gradient controller which offers the facility for connecting to a computer, a high precision two-head pump, a solenoid four-solvent delivery valve for solvent selection and mixing, a sample injection valve, and an absorbance detector to monitor column effluents. In contrast to the FPLC®, in the 650E system the liquid phase gradient is made at low pressure and prior to entering the pump.

Dye affinity adsorbents based on conventional supports result in low back pressures, when packed in short, wide columns to minimize particle deformation, can be run using peristaltic or other appropriate types of pumps. In contrast, adsorbents composed of small size particles suitable for HPAC (e.g. 5–10 μm) usually need to be run on high pressure instruments, such as those employed in HPLC applications. In this latter case, it is most advisable that titanium pump heads and tubings be used to prevent metal corrosion. However, small size particles with narrow size distribution (e.g. Monobeads®) can be run on medium pressure instruments (e.g. FPLC® and Waters 650E).

Introduction to Applications

Dyes were originally introduced as cheap alternatives to costly nucleotide ligands for enzyme purification. Given the advantages of dyes over biological ligands, their broad protein-binding spectrum, and also the possibility of improving their selectivity for targeted proteins via molecular modelling, it is no surprise that these colourful tools are finding increasing application in various affinity-based biosepartion techniques. The literature abounds with examples where dye ligands have been employed to purify individual proteins and enzymes: albumin, antibodies, blood-clotting factors, plasminogen activator, proteolytic activities, growth factors, interferons, cellulolytic and lipolytic enzymes, collagenases, restriction endonucleases, and numerous nucleotide-binding proteins and enzymes, to name some examples. It is no exaggeration to claim that for almost every protein purification problem, a dye ligand can be found to help towards its solution.

See also: **I/Affinity Separation. II/Affinity Separation:** Affinity Membranes; Affinity Partitioning in Aqueous Two-Phase Systems; Rational Design, Synthesis and Evaluation: Affinity Ligands. **Membrane Separations:** Membrane Bioseparations; Ultrafiltration. **III/Enzymes:** Liquid Chromatography. **Proteins:** Capillary Electrophoresis; Centrifugation; Electrophoresis. **Pressurised Fluid Extraction: Non-Environmental Applications. Appendix 1/Essential Guides for Isolation/Purification of Enzymes and Proteins; Appendix 2/Essential Guides to Method Development in Affinity Chromatography.**

Further Reading

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Hydrophobic Interaction Chromatography

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Introduction

According to J.N. Israelachvili (1985), hydrophobic interactions constitute 'the unusually strong attraction between nonpolar molecules and surfaces in water'. For two contacting methane molecules, the attraction energy is about sixfold higher in water than the van der Waals interaction energy in a vacuum. This energy, which has been estimated to be about -8.5 kJ mol^{-1} for two methane molecules is due to the extrusion of ordered water on two adjacent hydrophobic surfaces into less-ordered bulk water with a concomitant increase in entropy. This entropy-driven attraction between nonpolar groups in water is the basis for hydrophobic interaction chromatography.

The chromatographic separation of proteins depends on the differential accumulation of molecules at certain sites within a chromatographic system. Two principal types of chromatographic systems employing hydrophobic media have been described: (a) reversed-phase and (b) hydrophobic interaction chromatography. The principle of reversed-phase chromatography is based on a hydrophobic, e.g., silica, support of very high hydrophobicity which is capable of retaining nonpolar liquid phases (stationary liquid phase) when applied as the less polar phase in a solvent system. In this classical system the solutes are absorbed and separated (partitioned) in the apolar stationary liquid phase (i.e., a three-dimensional system) and not on the solid phase. In hydrophobic interaction chromatography the solutes (proteins) are adsorbed and separated on the apolar stationary solid phase (i.e., a two-dimensional system) carrying immobilized hydrophobic groups. Because of the very different scopes and methodological details, reversed-phase chromatography will not be treated here. The same holds for other forms of liquid–liquid partition chromatography. A differentiation will also not be made between classical chromatographic systems and HPLC since, in essence, it is only the bead or particle size which leads to the higher performance (e.g. throughput, resolution) in the latter method.

Discovery and Development of Hydrophobic Interaction Chromatography

The chromatographic purification of proteins on specifically synthesized hydrophobic solid supports was first reported independently by Yon and Shaltiel in 1972. In both cases the hydrophobic matrix consisted of agarose to which aminoalkane derivatives have been coupled by the CNBr method. Yon synthesized mixed hydrophobic-charged gels (aminodecyl-, or *N*-3-carboxypropionyl)aminodecyl-agarose) with an alkyl residue to charge ratio of at least 1 : 1 for the adsorption of lipophilic proteins such as bovine serum albumin or aspartate transcarbamoylase. These proteins were adsorbed at low ionic strength at the isoelectric point and eluted at acidic or alkaline pH by charge repulsion. The surprising result in Shaltiel's experiments was that a very normal hydrophilic enzyme, phosphorylase *b*, could be purified on hydrocarbon-coated agaroses to near homogeneity in one step, implicitly questioning the general doctrine of the time that all hydrophobic amino acids are buried in the interior of proteins. Phosphorylase was adsorbed at low ionic strength on immobilized butyl residues which had no resemblance to the substrates of the enzyme (excluding affinity chromatography) and was eluted by a 'deforming buffer' which imposed a limited conformational change on the enzyme. Taken together with Shaltiel's systematic approach of grading the hydrophobicity of the gels via an immobilized homologous hydrocarbon series, the immediate

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